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<p>(21) International Application Number: <b>PCT/GB97/02592</b></p> <p>(22) International Filing Date: <b>23 September 1997 (23.09.97)</b></p> <p>(30) Priority Data: <b>9619768.6 23 September 1996 (23.09.96) GB</b></p> <p>(71) Applicant (<i>for all designated States except US</i>): <b>THE UNIVERSITY COURT OF THE UNIVERSITY OF ST. ANDREWS [GB/GB]; 66 North Street, St. Andrews, Fife KY16 9AH (GB).</b></p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (<i>for US only</i>): <b>GANI, David [GB/GB]; Bois Feuris, Crail Road, St. Andrews, Fife KY16 8AP (GB). HILLIER, Mark [GB/GB]; 133B South Street, St. Andrews KY16 9UN (GB). HORMOZDIARI, Pan tea [IR/GB]; 19 All Saints Court, Didcot, Oxon OX11 7NG (GB).</b></p> <p>(74) Agent: <b>MURGITROYD &amp; COMPANY; 373 Scotland Street, Glasgow G5 8QA (GB).</b></p>		<p>(81) Designated States: <b>AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SI, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</b></p> <p><b>Published</b> <i>With international search report.</i></p>	
<p>(54) Title: <b>PHOSPHORYLATING REAGENTS</b></p> <p>(57) Abstract</p> <p>There is provided a phosphorylating reagent for phosphorylation of amino acids or compounds formed therefrom. The phosphorylating reagent is of utility in solution or solid-phase chemistry, and particularly for the solid-phase synthesis of phosphorylated peptides and combinatorial libraries of phosphorylated organic compounds. Also provided for is a method of phosphorylating oxygen, nitrogen and sulphur nucleophiles, for example amino acid and peptides.</p>			

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1       **Phosphorylating Reagents**

2

3       This invention describes novel phosphorylating reagents  
4       and the use thereof for solution or solid-phase  
5       chemistry and their particular use for the solid-phase  
6       synthesis of phosphorylated peptides and combinatorial  
7       libraries of phosphorylated organic compounds.

8

9       The reversible phosphorylation of proteins on serine,  
10      threonine and tyrosine residues, as catalysed by  
11      protein kinases and phosphatases, is the principal  
12      mechanism by which eukaryotic cells (the cells of  
13      multicellular organisms) respond to external stimuli<sup>1,2</sup>.  
14      Three groups of enzymes referred to collectively as the  
15      protein phosphatases (these enzymes hydrolyse the  
16      phosphoryl group of a phosphoprotein) are responsible  
17      for the dephosphorylation of the phosphoproteins. One  
18      group known as the serine-threonine protein  
19      phosphatases are collectively responsible for the  
20      dephosphorylation of certain phosphorylated serine or  
21      threonine residues within phosphoproteins (see Fig. 1A)  
22      and several different types exist (e.g. PP1, PP2A, PP2B  
23      and PP2C) most of which appear to be associated with  
24      regulatory proteins. A second group are referred to as  
25      the protein tyrosine phosphatases and these enzymes

1 hydrolytically remove the phosphoryl group from certain  
2 phosphotyrosine residues within phosphoproteins, Fig.  
3 1B. The third group of enzymes is responsible for  
4 removing the phosphoryl group from phosphohistidine  
5 residues within phosphoproteins, Fig. 1C.

6

7 Structure-activity studies for the phosphorylated  
8 peptide substrates of protein phosphatases have been  
9 limited by the availability of structurally diverse  
10 substrates because, to date, almost all of these have  
11 been prepared by enzymic phosphorylation using  
12 adenosine triphosphate (ATP) and appropriate protein  
13 kinase enzymes which are specific for certain  
14 sequences<sup>3</sup>. The specific nature of these enzymes  
15 restricts the scope of these studies in addition to the  
16 extra complications of separating the products of the  
17 reaction e.g. separating the phosphorylated peptide  
18 from adenosine monophosphate (AMP). Moreover, non-  
19 enzymic syntheses of phosphopeptides, in particular  
20 phosphothreonine peptides is severely hampered by  $\beta$ -  
21 elimination of phosphoric acid diester which occurs in  
22 synthetic intermediates to give the corresponding  
23 dehydroamino acid moieties<sup>4-6</sup>. Phosphothreonine peptide  
24 syntheses typically employ large excesses of highly  
25 electrophilic phosphorus (III) reagents to introduce  
26 phosphorus into the preformed peptide and then an  
27 oxidant (e.g. tertiary-butyl hydroperoxide) is required  
28 to convert the phosphite triester to the phosphate  
29 triester prior to deprotection of the ester groups<sup>4,5</sup>.  
30 While the peptide exists as its phosphate triester, it  
31 is particularly vulnerable to  $\beta$ -elimination, which is  
32 undesirable.

33

34 The existing methods for avoiding  $\beta$ -elimination in the  
35 synthesis of phosphoserine and phosphothreonine  
36 peptides involve introducing each of the phosphorylated

1      amino acid residues as their protected phosphate  
 2      diester monoanions<sup>6</sup>. These are however tedious to  
 3      prepare.

4

5      It is an object of the present invention to provide a  
 6      phosphorylating agent that would be electrophilic  
 7      enough to react directly and rapidly with primary and  
 8      secondary alcohol groups within resin-bound peptides.  
 9      Such agents would obviate the need for an oxidant, and  
 10     could possess labile phosphate ester protecting groups  
 11     that would be compatible with solid-phase peptide  
 12     synthesis.

13

14     This invention provides an electrophilic  
 15     phosphorylating reagent for amino acids and/or peptide  
 16     sequences thereof comprising a compound as represented  
 17     by formula (I):

18

19

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21

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25

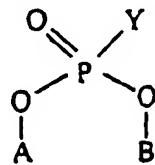
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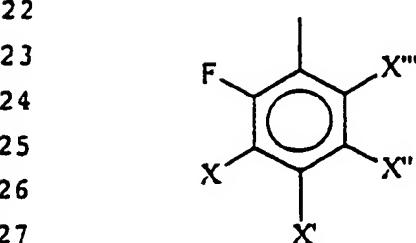
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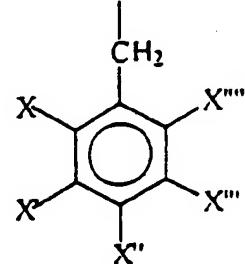
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(I)



(II)



(III)

31    wherein: A is a substituted aromatic group which is  
 32    represented by formula (II) e.g. a fluorophenyl or A is  
 33    an acid cleavable functionality such as a benzyl or  
 34    substituted benzyl group represented by formula (III);  
 35    B is a substituted aromatic group represented by  
 36    formula (II) e.g. a fluorophenyl group, but not a

1     benzyl or a substituted benzyl group;  
2     each X, X', X'', X''' and X'''' are individually H or F  
3     atoms or any suitable moiety;  
4     Y is any halogen or leaving group.

5

6     The leaving group Y is the group which does not contain  
7     the phosphorous atom following cleavage of compound I;  
8     for example Y can be Cl, Br, I, -NRR'R''(as a quaternary  
9     ammonium salt), -OR, -SR (wherein each group R, R' or R''  
10    is any group which does not affect the lability of the  
11    leaving group Y, especially under acidic and/or basic  
12    conditions, for example R, R' or R'' can individually be  
13    -H, -CH<sub>3</sub>, -C<sub>2</sub>H<sub>5</sub>, -C<sub>6</sub>H<sub>5</sub>, -C(O)-C<sub>1-12</sub> or -CH=C(OH)-C<sub>1-12</sub>).  
14

15    The reference to "any suitable moiety" with regard to  
16    groups X, X', X'', X''' and X'''' refers to any atom or  
17    group thereof which does not affect the lability of the  
18    compounds represented by formulae II or III.  
19

20    In one embodiment, the reagent is bis(tetrafluorophenyl)  
21    chlorophosphate; and in this embodiment the preferred  
22    reagent is bis (2, 3, 5, 6-tetrafluorophenyl)  
23    chlorophosphate.

24

25    In a further embodiment, the reagent is a benzyl,  
26    fluorophenyl halophosphate; and in this embodiment the  
27    preferred reagent is a benzyl, polyfluorophenyl  
28    chlorophosphate.

29

30    It can be seen that reagents represented by the formula  
31    (I) have a fluorine in at least one *ortho* position on at  
32    least one of the aromatic rings wherein the remaining  
33    positions X, X', X'' and X''' on (II) and X,

34

35    X', X'', X''' and X'''' on (III) can each be -H or -F  
36    atoms or any suitable moiety in any permutation.

1

2     Additionally, the -H atom or -F atom or suitable moiety  
3     may be in the presence or absence of one or more  
4     similar or dissimilar other ring substituents.

5

6     A further embodiment has a halogen or other leaving  
7     group attached to the phosphorus atom of reagent (I) at  
8     Y, where the leaving group can be one of -OR, -NRR',  
9     -NRR'R'' or -SR, wherein R, R' and R'' can be any  
10    suitable moiety.

11

12    The invention further provides a method for the  
13    phosphorylation of oxygen, nitrogen or sulphur  
14    nucleophiles of amino acids and/or peptides wherein the  
15    nucleophile is treated with an excess of a reagent of  
16    general formula (I) followed by hydrolysis of the  
17    product.

18

19    Preferably the hydrolysis reagent is trifluoroacetic  
20    acid.

21

22    The oxygen nucleophile may be part of a primary or  
23    secondary alcohol, phenol, carboxylate or enolate  
24    group.

25

26    The amino acids may be present as single species or in  
27    combination within or outwith the same molecule, as in  
28    peptide sequences.

29

30    Suitably, the amino acid(s) may be tyrosine, serine and  
31    threonine.

32

33    In one particular embodiment of the invention, the  
34    amino acid is present as a resin bound moiety.

35

36    In further embodiments of the invention, the

1 phosphorylation method may be utilised in solid, liquid  
2 or gel phase.

3  
4 The method is of considerable potential in the solid-  
5 phase synthesis of a whole range of organic phosphates  
6 from primary and secondary alcohols and phenols and is  
7 completely compatible with combinatorial and  
8 permutational organic synthesis.

9  
10 In the area of peptide chemistry the method offers very  
11 significant advantages over the previously used two  
12 step phosphorylation-oxidation strategies,  
13 furthermore, the use of bis-(pentafluorophenyl)  
14 chlorophosphate (11) is of particular utility in the  
15 preparation of peptides containing two or more  
16 phosphorylated residues via a "global phosphorylation"  
17 strategy which involves introducing all of the  
18 phosphoryl groups in one step after the synthesis of  
19 the required peptide. The same is true for the  
20 introduction of more than one phosphoryl group into  
21 other organic molecules which contain more than one  
22 alcohol and/or phenol group.

23  
24 The examples illustrate that primary alcohols,  
25 secondary alcohols and phenols whether present as  
26 single species, or in combination within or outwith the  
27 same molecule, are efficiently phosphorylated by the  
28 polyfluoroaromatic chlorophosphate reagents. Other  
29 oxygen nucleophiles, for example, carboxylate and  
30 enolate, and other nucleophiles, for example, those  
31 derived from nitrogen and sulphur are also expected to  
32 react with similar efficiency with the reagent.

33  
34 The examples herein relate to the phosphorylation  
35 reaction by bis-(pentafluorophenyl chlorophosphate (11)  
36 and other polyfluoroaromatic halophosphates shown by

1 general formula I, where any, some or all X groups is H  
2 and/or F or other suitable moiety in any permutation  
3 whether in the presence or absence of one or more  
4 similar or dissimilar other ring substituents; (Y is a  
5 halogen or other leaving group) which should effect a  
6 similar facile phosphorylation. Furthermore,  
7 triesters, derived from oxygen nucleophiles, or any  
8 other phosphorylated derivative containing the  
9 polyfluoroaromatic phosphate diester protection;  
10 wherein Y = -OR, -NRR', -NRR'R'', -SR, (where each group  
11 R, R' or R'' can be any suitable moiety as defined  
12 above) should be more labile to deprotection under  
13 acidic conditions (and/or under basic conditions) than  
14 the corresponding bis-phenyl phosphate diester  
15 protection.

16

17 This method provides higher yields of phosphorylated  
18 product of high quality with less or no wasteful side  
19 reactions.

20

21 This invention is further described in a non-limiting  
22 manner by reference to the following examples and  
23 accompanying figures wherein:

24

25 Fig. 1a Illustrates the enzymatic dephosphorylation  
26 of a phosphorylated threonine (or serine)  
27 residue.

28

29 Fig. 1b Illustrates the enzymatic dephosphorylation  
30 of a phosphorylated tyrosine residue.

31

32 Fig. 1c Illustrates the enzymatic dephosphorylation  
33 of a phosphorylated histidine residue.

34

35 Fig. 2: Illustrates reaction schemes 1A & 1B.  
36 Reagents and Conditions: i) 20%

1                   piperidine/DMF; ii) 5% (CH<sub>3</sub>CO)<sub>2</sub>O/DMF; iii)  
2                   DMAP, TEA, PO(OPh)<sub>2</sub>Cl, DCM, 20°C; iv) 82.5%  
3                   TFA: 5% phenol: 5% H<sub>2</sub>O: 5% thioanisole; 2.5%  
4                   EDTA (reagent K), 80%; v) LiOH (aq),  
5                   EtOH/CH<sub>3</sub>CN; vi) DMAP, TEA, PO(OPhF<sub>5</sub>)<sub>2</sub>Cl, DCM,  
6                   20°C; vii) Dowex Cl, 60%.

7  
8       Fig. 3a: Shows the structure of bis(pentafluorophenyl)  
9                   chlorophosphate (11).

10  
11      Fig. 3b: Shows the structure of the  
12                   bis(pentafluorophenyl) phosphate derivative  
13                   of cyclohexanol (12).

14  
15      Fig. 4: Shows the structure of pentafluorobenzyl-  
16                   pentafluorophenyl chlorophosphate (13).

17  
18      Fig. 5: Illustrates reaction scheme 2. Reagents and  
19                   Conditions: i) 1.01 eq N-Chlorosuccinimide,  
20                   toluene, 2hr, rt; ii) NaH, C<sub>6</sub>F<sub>5</sub>OH, THF, 1hr,  
21                   rt; iii) a) NaI, acetone, Δ, 15 mins. b)  
22                   HCl<sub>(aq)</sub>; iv) PCl<sub>5</sub>, DCM.

23  
24      Fig. 6a: Shows the structure of the benzyl  
25                   pentafluorophenyl derivative of cyclohexanol  
26                   (18).

27  
28      Fig. 6b: Shows the structure of the benzyl  
29                   pentafluorophenyl derivative of N-α-<sup>t</sup>Boc-  
30                   tyrosine methyl ester (19).

31  
32      Fig. 6c: Shows the structure of the phosphopeptide  
33                   Asp-Ala-Asp-Glu-Tyr(OPO<sub>3</sub>H<sub>2</sub>)-Leu (23).

34  
35      Fig. 7: Illustrates reaction scheme 3. Reagents and  
36                   Conditions: i) 20% piperidine/DMF; ii) DMAP,

1           TEA, PO(OCH<sub>2</sub>Ph)(OPhF<sub>3</sub>), DCM, 20°C; iii) NaOH  
2           (aq), DMSO; iv) 90% TFA, 5% H<sub>2</sub>O, 5% Et<sub>3</sub>SiH.

3

4           Example 1

5

6           Diphenyl chlorophosphate had been successfully employed  
7           to phosphorylate the secondary alcohol groups of myo-  
8           inositol and its analogues<sup>7</sup>. Using an N-acetyl (Ac)  
9           capped analogue of a known consensus sequence for a  
10          PP2A substrate as the target, AcNH-Arg-Arg-Ala-  
11          Thr(PO<sub>3</sub>H<sub>2</sub>)-Val-Ala-OH (1), a series of solid-phases  
12          phosphorylation reactions were examined. Accordingly,  
13          using Wang resin, standard Fmoc chemistry with PyBOP  
14          activation, and arginine residue precursors containing  
15          2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc)  
16          protected guanidino groups, the peptide Fmoc-NH-Arg-  
17          Arg-Ala-Thr-Val-Ala-O-Wang (2) was prepared. The N-  
18          terminal Fmoc group was removed with 20% piperidine in  
19          DMF and the free amino group was capped with 5% acetic  
20          anhydride in DMF to give compound (3). Treatment of  
21          the resin-bound peptide (3) with diphenyl  
22          chlorophosphate gave some of the required diphenyl  
23          threonine phosphate triester (4), and under optimised  
24          conditions (repeated treatments with 20 equivalents of  
25          diphenyl chlorophosphate in the presence of DMAP and  
26          TEA for 6-8 hours at ambient temperature) essentially  
27          quantitative conversion to the triester (4) could be  
28          achieved, as determined by NMR-spectroscopic analysis  
29          of the products after cleavage from the resin Fig. 2,  
30          Scheme 1A.

31

32          <sup>1</sup>H-, <sup>13</sup>C- and <sup>31</sup>P-NMR spectra showed the expected  
33          signals, chemical shift changes and P-C and P-H  
34          couplings for the required triester (5). All attempts  
35          to hydrolyse the pure triester (5) under mild basic  
36          conditions resulted in the formation of significant

1       quantities of the  $\beta$ -elimination product,  
2       dehydrobutyrine peptide (6), as judged by  $^1\text{H}$ - and  $^{31}\text{P}$ -  
3       NMR spectroscopy.

4

5       Example 2

6

7       In order to increase the electrophilicity at phosphorus  
8       in the phosphorylating species (to decrease reaction  
9       times) and also in the required peptide phosphate  
10      triester (to facilitate deprotection), the preparation  
11      and use of bis-(pentafluorophenyl) chlorophosphate (11)  
12      was investigated. The reagent was prepared by treating  
13      phosphorus oxychloride (7) with 1.8 equivalents of  
14      pentafluorophenol (8) at 140°C for 16-24 hours and was  
15      purified by removing the unreacted starting materials  
16      by distillation. The resulting reagent (11) was 85-90%  
17      pure as judged by  $^{19}\text{F}$ - and  $^{31}\text{P}$ -NMR spectroscopy and could  
18      be further purified by fractional distillation.

19



21

22       Example 3

23

24       In model reactions using cyclohexanol, the bis-  
25       (pentafluorophenyl) chlorophosphate (11) reacted at  
26       least 30-fold more rapidly than diphenyl  
27       chlorophosphate to give the required triester (12)  
28       which was fully characterised. Note that the bis-  
29       (2,3,5,6-tetrafluorophenyl) chlorophosphate analogue of  
30       reagent (11), which was more useful for mechanistic  
31       studies and for product characterisation (due to the  
32       presence of an integratable proton resonance in  $^1\text{H}$ -NMR  
33       spectra), behaved similarly in effecting rapid  
34       phosphorylation.

35

36

1    Example 4

2

3    Treatment of the Pmc protected resin-bound peptide, Ac-  
4    NH-Arg-Arg-Ala-Thr-Ala-Val-Ala-O-Wang(3), with 10  
5    equivalents of bis-(pentafluorophenyl) chlorophosphate  
6    under optimised conditions gave the resin-bound  
7    phosphate triester (9) in excellent yield, Scheme 2B.  
8    Immediate deprotection of the two Pmc groups, the two  
9    pentafluorophenyl groups, and simultaneous cleavage  
10   from the resin occurred upon treatment with aqueous  
11   trifluoroacetic acid solutions to give the almost pure  
12   N-capped phosphorylated threonine peptide (10) in  
13   essentially quantitative conversion. There was no  
14   evidence whatsoever for  $\beta$ -elimination products and  
15   purification on Dowex 1 chloride (Trade Mark) gave the  
16   pure phosphopeptide (10) in 60% overall yield (over 14  
17   solid-phase steps). This material was fully  
18   characterised and served as a substrate for protein  
19   phosphatase  $\lambda$  as judged by directly monitoring the  
20   course of phosphopeptide hydrolysis by  $^1\text{H-NMR}$   
21   spectroscopy.

22

23    Example 5

24

25    Other peptides containing serine residues or tyrosine  
26    residues were also successfully phosphorylated with  
27    bis-(pentafluorophenyl) chlorophosphate (11) using  
28    similar protocols.

29

30    Example 6

31

32    Merrifield resin bound inositol analogues, connected by  
33    ether linkages which are stable to trifluoroacetic  
34    acid, were successfully phosphorylated on secondary  
35    alcohol moieties by bis-(pentafluorophenyl)  
36    chlorophosphate (11) using similar protocols.

1 Treatment with aqueous trifluoroacetic acid resulted in  
2 the deprotection of the pentafluorophenyl groups to  
3 give resin bound inositol monophosphate analogues.  
4 ( $\delta_p$  (121.41 MHz,  $C_6^{2}H_6$ ): -10.443).

5

6 Example 7

7

8 In both solution and solid phase phosphorylations of  
9 phenols it was noted that, whilst the actual  
10 phosphorylation reaction with bis-(pentafluorophenyl)  
11 chlorophosphate (11) was facile, complete removal of  
12 the pentafluorophenyl groups was difficult. In each  
13 case, the first pentafluorophenyl group could be  
14 removed easily in the presence of trifluoroacetic acid  
15 solution, but the second pentafluorophenyl group could  
16 not. Therefore, since it appeared that the acidity of  
17 the partially deprotected phosphoric acid derivative  
18 was too high for protonation by the trifluoroacetic  
19 acid solution, modified reagents were designed, (for  
20 example preferably formula I, where II is a substituted  
21 phenyl group (where X, X', X'', X''' are H or F atoms  
22 or any suitable moiety), III is a benzyl or substituted  
23 benzyl group (where X, X', X'', X''', X'''' are H or F  
24 atoms or any suitable moiety) but not a phenyl or  
25 substituted phenyl group, and Y is any halogen. It was  
26 expected that the phenyl or substituted phenyl group  
27 (derived from the reagent) of the intermediate triester  
28 (phosphorylated alcohol or phenol) would be removed in  
29 a facile manner by base catalysed hydrolysis, and that  
30 the benzyl or substituted benzyl group could be removed  
31 in a facile manner by acid catalysed hydrolysis,  
32 preferably in the presence of trifluoroacetic acid,  
33 which would be compatible with other solid state  
34 synthesis protocols.

35

36 To prepare such substituted phenyl substituted benzyl

1       halophosphates, a model synthetic protocol was  
2       developed using benzyl pentafluorophenyl chlorophosphate  
3       as the target (Scheme 2).

4

5       Treatment of dibenzyl phosphite (14) with *N*-  
6       chlorosuccinimide in toluene<sup>8</sup>, followed by reaction with  
7       sodium pentafluorophenolate (formed by the reaction  
8       between sodium hydride and pentafluorophenol in THF)  
9       resulted in the formation of dibenzyl pentafluorophenyl  
10      phosphate triester (15). <sup>1</sup>H, <sup>13</sup>C, <sup>19</sup>F and <sup>31</sup>P-NMR spectra  
11      showed the expected chemical shift changes and P-C and  
12      P-H coupling constants consistent with those expected  
13      for the required triester.  $\delta_H$ (300 MHz, C<sup>2</sup>HCl<sub>3</sub>): 5.21 (d,  
14      J<sub>PH</sub> 8.7, CH<sub>2</sub>OP),  $\delta_c$ (75.4 MHz, C<sup>2</sup>HCl<sub>3</sub>): 70.88 (d, CH<sub>2</sub>OP, J<sub>PC</sub>  
15      6.5),  $\delta_p$ (121.41 MHz, C<sup>2</sup>HCl<sub>3</sub>): -5.44, and the correct  
16      mass ion (*m/z*(CI+ mode) 444, M<sup>+</sup> molecular ion).

17

18      Reaction of the triester (15) with 1 equivalent of  
19      anhydrous sodium iodide in refluxing acetone for 15  
20      minutes gave a white solid, which upon cooling was  
21      isolated by filtration, then dissolved in water and  
22      treated with aqueous hydrochloric acid.<sup>9</sup> The resulting  
23      precipitate of benzyl pentafluorophenyl phosphoric acid  
24      diester (16) was isolated in essentially quantitative  
25      yield from the dibenzyl pentafluorophenyl phosphate  
26      triester (15). <sup>1</sup>H, <sup>13</sup>C, <sup>19</sup>F and <sup>31</sup>P-NMR spectra showed  
27      the expected chemical shift changes and P-C and P-H  
28      coupling constants for the required diester.  $\delta_H$ ,(300  
29      MHz, C<sup>2</sup>HCl<sub>3</sub>): 5.20 (2H, d, J<sub>PH</sub> 8.4, CH<sub>2</sub>OP),  $\delta_c$ (75.4 MHz,  
30      C<sup>2</sup>HCl<sub>3</sub>): 70.92 (d, CH<sub>2</sub>OP, J<sub>PC</sub> 5.4),  $\delta_p$ (121.41 MHz, C<sup>2</sup>HCl<sub>3</sub>):  
31      -4.66. Mass spectrometry confirmed the desired product  
32      had been obtained (*m/z* (EI+ mode): 354 (M<sup>+</sup> molecular  
33      ion)). Reaction of benzyl pentafluorophenyl phosphoric  
34      acid diester (16) with an excess of PCl<sub>5</sub> in  
35      dichloromethane followed by removal of the solvent at  
36      reduced pressure (20mm/Hg) and separation of the by-

1 products by distillation at 0.1 mm Hg/30-40°C afforded  
2 the reagent (17) in better than 75% purity as judged by  
3  $^1\text{H}$  and  $^{31}\text{P}$ -NMR.  $\delta_{\text{H}}$ (300 MHz,  $\text{C}^2\text{HCl}_3$ ): 5.38 (2H, d,  $J_{\text{PH}}$  9.9,  
4  $\text{CH}_2\text{OP}$ ),  $\delta_{\text{C}}$ (75.4 MHz,  $\text{C}^2\text{HCl}_3$ ): 72.91 (d,  $\text{CH}_2\text{OP}$ ,  $J_{\text{PC}}$  7.54),  
5  $\delta_{\text{P}}$ (121.41 MHz,  $\text{C}^2\text{HCl}_3$ ): main peak at -2.39. Mass  
6 spectrometric analysis also gave the expected data ( $m/z$   
7 (EI $^+$ ): 372, 374 (Cl isotopes,  $\text{M}^+$  molecular ion). The  
8 reagent was found to be unstable at high temperatures  
9 (50°C) and decomposed if heated for prolonged periods  
10 above that temperature. The major contaminant  
11 displayed 2 signals at -18.6 and -19.5 ppm in the  $^{31}\text{P}$   
12 NMR spectrum of the product and corresponding signals  
13 in the  $^1\text{H}$ ,  $^{13}\text{C}$  and  $^{19}\text{F}$  NMR spectra, consistent with the  
14 expected properties of the bis-(benzyl)-bis-  
15 (pentafluorophenyl) pyrophosphate. The mass spectrum  
16 of the contaminant showed a molecular fragment ( $m/z$   
17 (CI $^+$ ) 507,  $[\text{M}-\text{OPhF}_5]^+$ ) consistent with the structure of  
18 the pyrophosphate. Since this material would give  
19 identical phosphorylated products to the  
20 chlorophosphate, the crude reagent was used routinely  
21 for solid phase phosphorylations.

22  
23 Other benzyl phenyl chlorophosphates were prepared  
24 using analogous methods.

25

26 Example 8

27

28 In model phosphorylation reactions in solution using  
29 cyclohexanol, the benzyl pentafluorophenyl  
30 chlorophosphate (17) reacted with cyclohexanol in the  
31 presence of triethylamine in dichloromethane to give  
32 the required triester (18). This was characterised by  
33  $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{19}\text{F}$  and  $^{31}\text{P}$ -NMR spectroscopy and gave the  
34 expected data.

35

36

1    Example 9

2

3    In model phosphorylation reactions in solution using  
4    N-<sup>t</sup>Boc-(2S)-tyrosine methyl ester, the benzyl  
5    pentafluorophenyl chlorophosphate (17) reacted with the  
6    phenolic hydroxyl group in the presence of  
7    triethylamine in dichloromethane to give the required  
8    triester(19). This was characterised by <sup>1</sup>H, <sup>13</sup>C, <sup>19</sup>F and  
9    <sup>31</sup>P-NMR spectroscopy and mass spectrometry and gave the  
10   expected data.

11

12   Example 10

13

14   In model solid state phosphorylation reactions,  
15   treatment of the resin-bound peptide Fmoc-Val-Tyr-Leu-  
16   O-Wang (20) with 10 equivalents of freshly prepared  
17   benzyl pentafluorophenyl chlorophosphate (17) under  
18   optimised conditions gave the resin bound phosphate  
19   triester (21) in excellent yield, Scheme 3. Treatment  
20   with 20% piperidine in DMF removed the N-terminal Fmoc  
21   group. Subsequent treatment of the product with an  
22   excess of 1 mol.dm<sup>-3</sup> aqueous NaOH in DMSO followed by  
23   washing and treatment with aqueous trifluoroacetic acid  
24   resulted in deprotection of the pentafluorophenyl and  
25   benzyl groups and cleavage of the resin C-terminal  
26   ester linkage to give the phosphopeptide Val-  
27   Tyr(OPO<sub>3</sub>H<sub>2</sub>)-Leu (23), δ<sub>p</sub> (121.41 MHz, <sup>2</sup>H<sub>2</sub>O):-3.42.

28

29   Example 11

30

31   Treatment of the tris-tert-butyl ester protected resin  
32   bound peptide Fmoc-NH-Asp(O<sup>t</sup>Bu)-Ala-Asp(O<sup>t</sup>Bu)-Glu(O<sup>t</sup>Bu)-  
33   Tyr-Leu-O-Wang in a similar manner to that described in  
34   Example 10 above afforded the almost pure hexapeptide  
35   Asp-Ala-Asp-Glu-Tyr(OPO<sub>3</sub>H<sub>2</sub>)-Leu (24) which showed the  
36   expected NMR spectroscopic data. This product

1 corresponds to the structure of the autophosphorylation  
2 site of the epidermal growth factor receptor (EGFR)<sup>10</sup> in  
3 its phosphorylated form.

4

5 Example 12

6

7 Treatment of the resin bound and protected peptide Ac-  
8 NH-Arg(Pmc)-Arg(Pmc)-Ala-Thr-Val-Ala-O-Wang (3) with 10  
9 equivalents of benzyl pentafluorophenyl chlorophosphate  
10 (17) under optimised conditions gave the benzyl  
11 pentafluorophenyl peptide phosphate triester.

12 Treatment of the resulting triester overnight with an  
13 excess of 1 mol.dm<sup>-3</sup> aqueous NaOH in DMSO followed by  
14 washing and subsequent treatment with aqueous  
15 trifluoroacetic acid resulted in deprotection of the  
16 two 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc)  
17 groups, the pentafluorophenyl and benzyl groups and  
18 cleavage of the C-terminal resin ester moiety to give  
19 the almost pure N-capped phosphohexapeptide (10).  
20 Spectroscopic data showed this material to be identical  
21 to that prepared in Example 4 above.

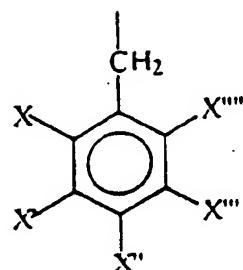
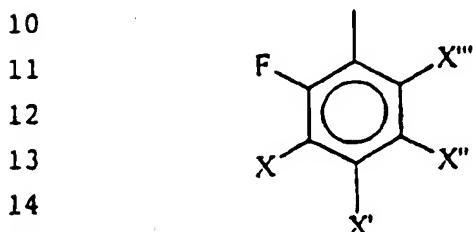
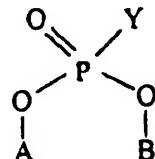
22

23 The serine analogue of (10) was prepared using a  
24 similar protocol.

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36                  90.

1 Claims

2  
3 1. An electrophilic phosphorylating reagent for amino  
4 acids and/or peptide sequences thereof comprising  
5 of a compound represented by formula (I) wherein:



(11)

(III)

18           A is a substituted aromatic group which is  
19           represented by formula (II) or A is an acid  
20           cleavable functionality such as a benzyl or  
21           substituted benzyl group represented by formula  
22           (III);  
23

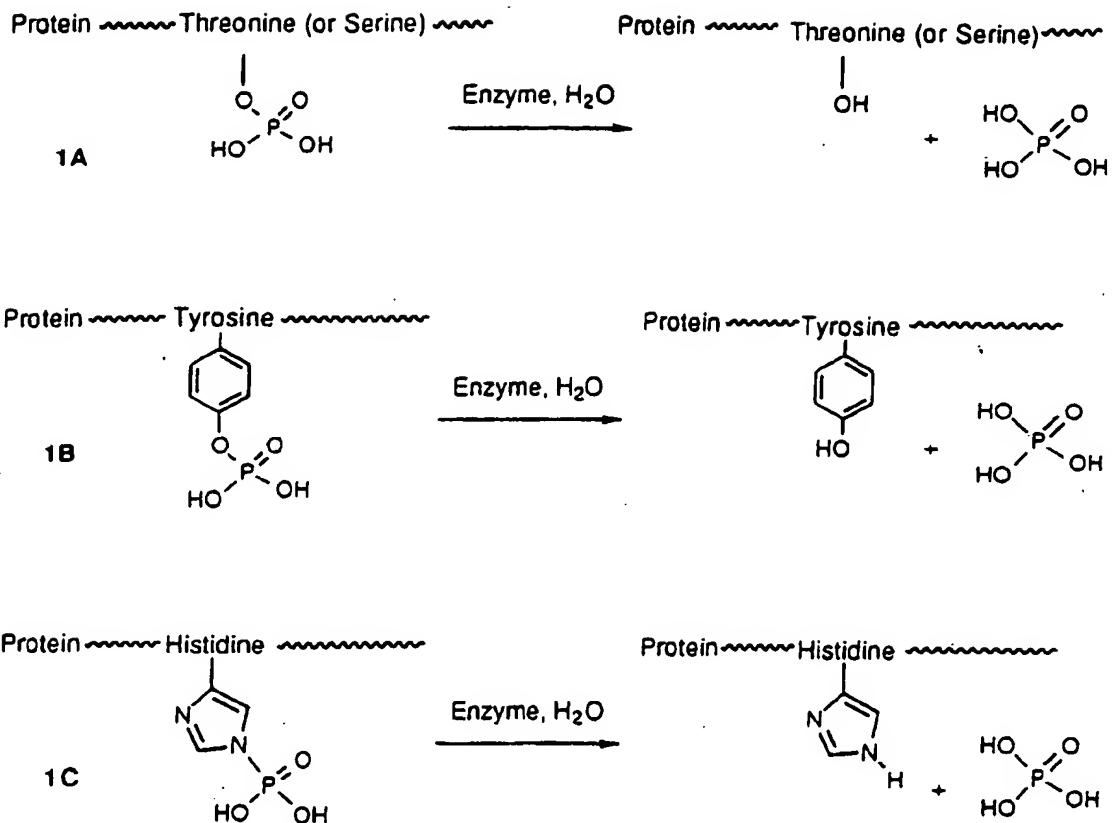
24 B is a substituted aromatic group represented by  
25 formula (II); X, X', X'', X''' and X'''' are each  
26 H or F atoms or any suitable moiety;  
27 Y is a halogen or leaving group.

28  
29     2. A phosphorylating reagent as claimed in Claim 1,  
30       wherein at least one of the compounds represented  
31       by formulae (II) and (III) is fully substituted by  
32       fluorine.

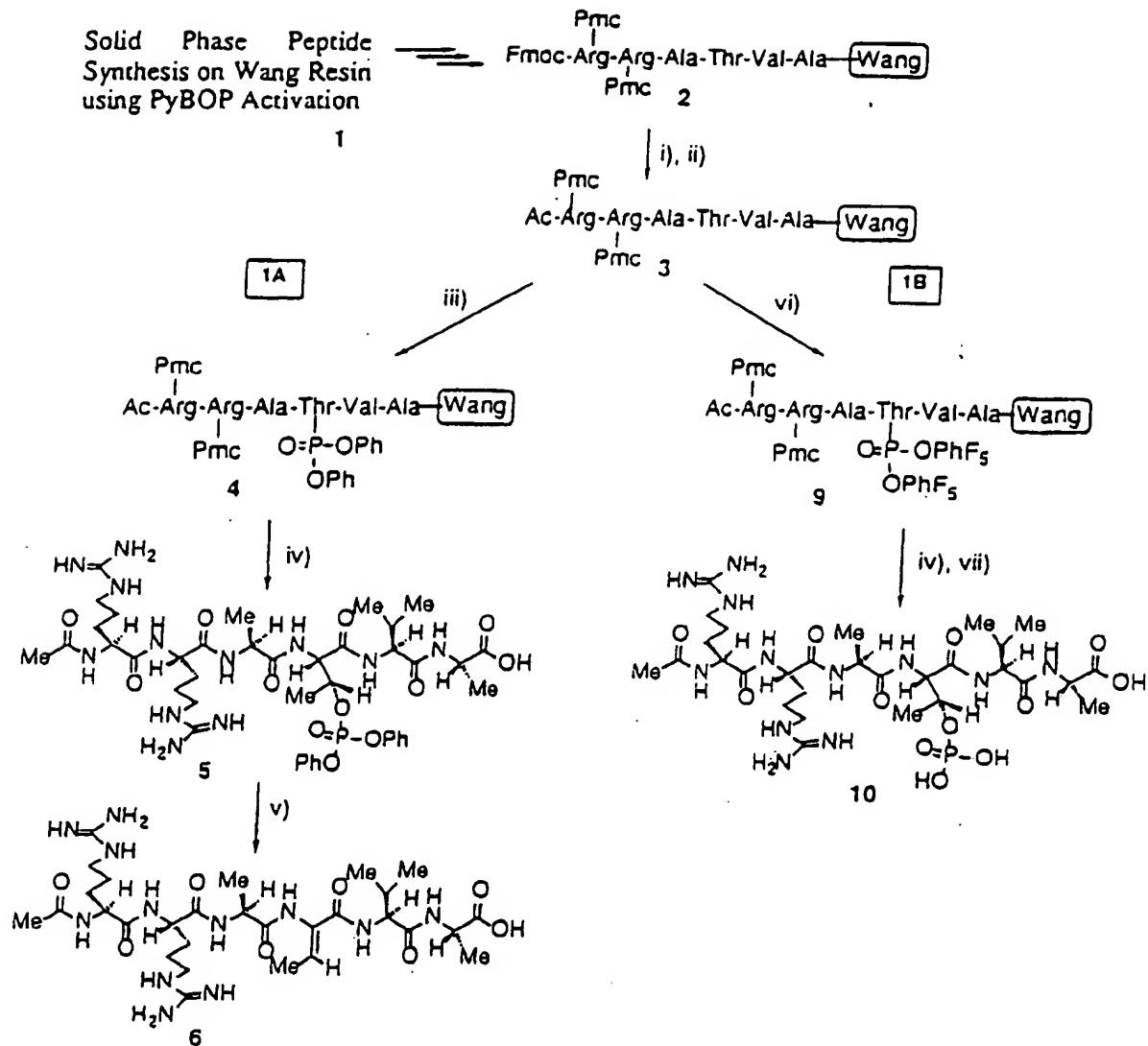
34      3. A phosphorylating reagent as claimed in either of  
35                Claims 1 and 2 wherein group Y is a chlorine atom.

- 1       4. A phosphorylating reagent as claimed in any one of  
2           Claims 1 to 3 which is bis(pentafluorophenyl)  
3           chlorophosphate .  
4
- 5       5. A phosphorylating reagent as claimed in Claim 3  
6           which is bis (2, 3, 5, 6 - tetrafluorophenyl)  
7           chlorophosphate.  
8
- 9       6. A phosphorylating reagent as in any one of Claims  
10          1 to 3 where the reagent is a benzyl, fluorophenyl  
11          halophosphate.  
12
- 13      7. A phosphorylating reagent as in claim 6 where the  
14          reagent is a benzyl, polyfluorophenyl  
15          chlorophosphate.  
16
- 17      8. A method for the phosphorylation of oxygen,  
18          nitrogen or sulphur nucleophiles of amino acids  
19          and/or compounds comprising an amino acid-like  
20          moiety wherein the nucleophile is treated with an  
21          excess of a reagent of general formula (I) as  
22          defined in Claim 1 followed by the hydrolysis of  
23          the product.  
24
- 25      9. A method as in claim 8 where the oxygen  
26          nucleophile may be part of a primary or secondary  
27          alcohol, phenol, carboxylate or enolate group.  
28
- 29      10. A method as in either one of claims 8 and 9 where  
30          the amino acids may be present as single species  
31          or in combination within or outwith the same  
32          molecule, as in peptide sequences.  
33
- 34      11. A method as in claim 10 where the amino acid(s)  
35          may be tyrosine, serine and threonine.  
36

- 1       12. A method as in any one of claims 8 to 11 where the
  - 2              amino acid and/or peptide is present as a resin
  - 3              bound moiety.
  - 4
  - 5       13. A method as in any one of claims 8 to 12 where the
  - 6              phosphorylation method may be utilised in solid,
  - 7              liquid or gel phase.
  - 8
  - 9       14. A method as in any one of claims 8 to 13 where the
  - 10             hydrolysis reagent is trifluoroacetic acid.



## FIGURE 1



**FIGURE 2**

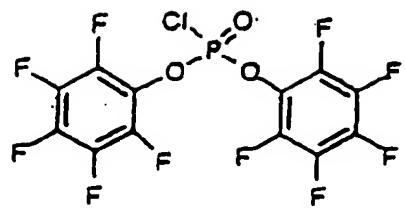


FIGURE 3A

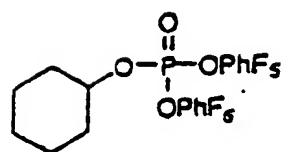
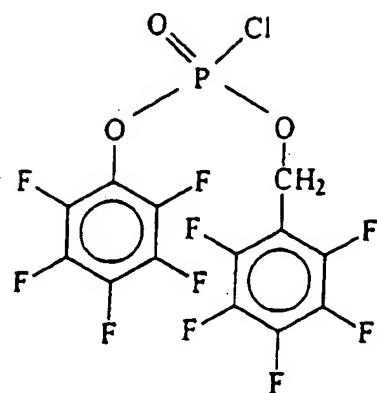


FIGURE 3B

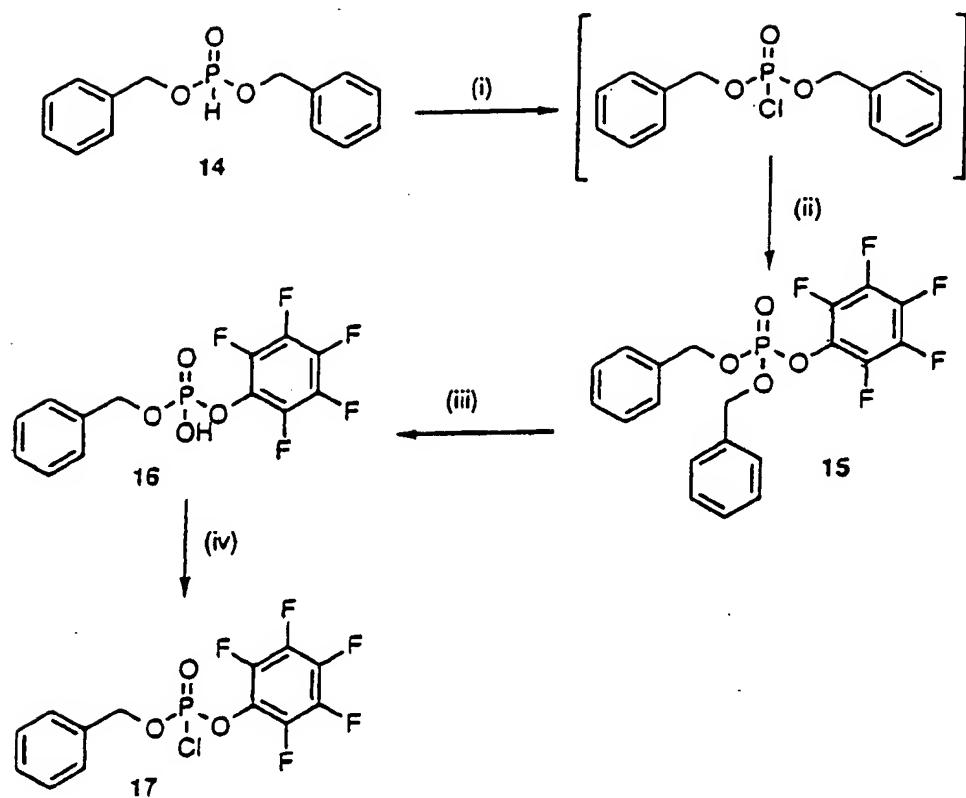


13

FIGURE 4

4/7

FIGURE 5



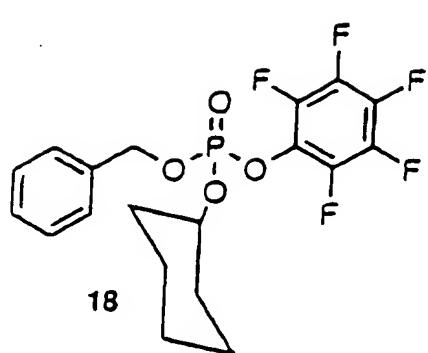


FIGURE 6A

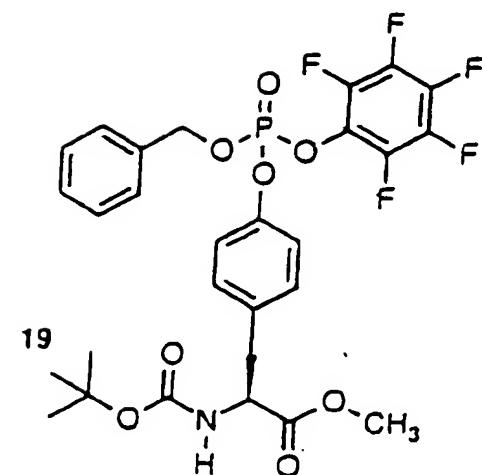


FIGURE 6B

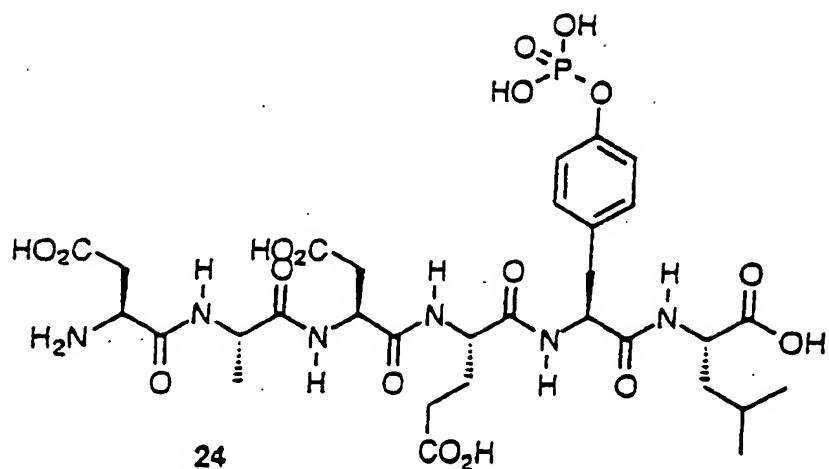
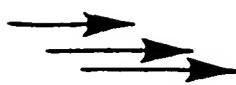


FIGURE 6C

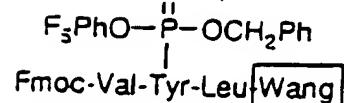
Solid Phase Peptide Synthesis  
on Wang Resin using Pybop  
Activation



Fmoc-Val-Tyr-Leu[Wang]

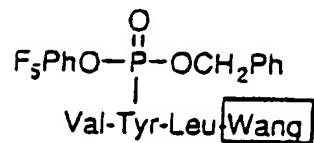
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i)



21

ii)



22

iii), iv)

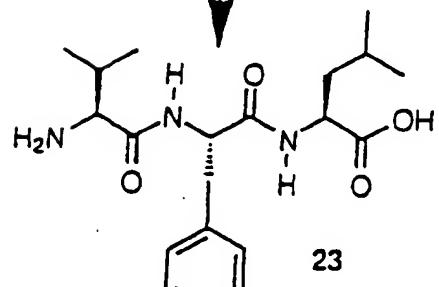


FIGURE 7

# INTERNATIONAL SEARCH REPORT

Inte  final Application No

PCT/GB 97/02592

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC 6 C07F9/14 C07K1/00 C07K1/04 C07F9/12

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C07F C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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X	US 3 341 630 A (ROBERT H. BOSCHAN) 12 September 1967 see the whole document ---	1-4
X	US 3 341 631 A (CHRISTIAN A. SEIL) 12 September 1967 see the whole document ---	1,2
X	US 3 408 427 A (ROBERT H. BOSCHAN) 29 October 1968 see the whole document ---	1,3
Y	US 5 245 069 A (JAMES W. McMANUS) 14 September 1993 see the whole document ---	1-14 -/-

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Y	SCHULZ J ET AL: "Synthesis and properties of mechanism-based inhibitors and probes for inositol monophosphatase derived from 6-O-(2'-hydroxyethyl)-(1R,2R,4R,6R)-cyclohexane-1,2,4,6-tetraol" J. CHEM. SOC., CHEM. COMMUN. (JCCCAT,00224936);95; (22); PP.2353-6, THE UNIVERSITY, ST. ANDREWS; SCHOOL CHEMISTRY; FIFE; KY16 9ST; UK (GB), XP002047053 cited in the application see the whole document ---	1-14
Y	JAN HES: "Di(2-tert-butylphenyl) Phosphorochloride. A new selective Phosphorylating agent." JOURNAL OF ORGANIC CHEMISTRY., vol. 39, no. 25, 1974, EASTON US, pages 3767-3769, XP002047054 see the whole document ---	1-14
P,X	HORMOZDIARI P ET AL: "Highly efficient solid-phase phosphopeptide synthesis using bis(polyfluorophenyl) chlorophosphates: preparation of serine-threonine protein phosphatase substrates" TETRAHEDRON LETT. (TELEAY,00404039);96; VOL.37 (45); PP.8227-8230, THE UNIVERSITY; SCH. CHEM.; ST. ANDREWS, FIFE; KY16 9ST; UK (GB), XP002047055 see the whole document ---	1-14

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

International Application No  
**PCT/GB 97/02592**

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 3341630 A	12-09-67	NONE	
US 3341631 A	12-09-67	NONE	
US 3408427 A	29-10-68	NONE	
US 5245069 A	14-09-93	NONE	